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An efficient isolation method for high-quality DNA from ancient bones

Michael Scholz[a] and Carsten Pusch[b]

[a]Michael Scholz, Institute of Prehistorical Research, Department of Archaeobiology, University of Tuebingen, Eugenstrasse 40, D-72072 Tuebingen, Germany
[b]Carsten Pusch, Institute of Anthropology and Human Genetics, Department of Molecular Genetics, University of Tuebingen, Wilhelmstrasse 27, D-72074 Tuebingen, Germany

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Many scientific investigations in the field of palaeogenetics are limited by the poor quality as well as the small quantity of genomic DNA extracted from prehistoric bones (Ref. 1, 2, 3). PCR reactions using mitochondrial (mt)-specific primers are commonly used to obtain sequence information from prehistoric DNA (Ref. 4, 5, 6). In previous studies, however, amplification of episomal DNA such as the hypervariable D-loop control region C was a compromise based on the large number of copies of mt sequences. More material of better quality is needed in order to perform PCR reactions using the short tandem repeat (STR) systems in use in present-day forensic medicine. In order to fulfill these requirements, we developed a preparation method which produces prehistoric human DNA of high quality extremely efficiently.

When taking samples we took the following precautions to prevent possible contamination. All stages of the work were carried out under sterile conditions, using latex gloves, mouth masks and plexiglass face masks. All appliances and containers used for working with or storing the bone material were cleaned to remove any remaining bone meal residue in several steps (stone meal, acetone, propan-2-ol) before and after use. Irradiation of the work area with UV-C light for approximately 2 h removed all DNA fragments. A two-stage decontamination of the containers and instruments used was carried out using substances capable of dissolving DNA and DNAses DNAaway (Molecular BioProducts Inc.).

Only certain areas of prehistorical skeletal remains are suitable for DNA extraction. We mainly took samples from the diaphysis region of the long extremity bones such as femur, tibia and humerus. These consist of spongious substances, which are unsuitable for use in the subsequent extraction, and the Compacta, a solid bone substance which, because of its dense structure and when preserved under favourable conditions, contains a sufficient amount of organic components (collagens) from which DNA can be isolated.

Preparation of the bone samples

The total sample consists of 2 or 3 bone fragments of a length of 1–2 cm; several fragments are needed because storage conditions can affect the degree of DNA preservation. The bone parts are removed using a hand mill (DBP Supra; Kaltenbach & Voigt/Biberach) that is normally used in dental work. For increased precision, the mill was fitted with a diamond separation disc (Orthodontros, Ultraflex 912-EF).

After the fragments have been removed, a slice of about 3 mm is milled off the sides of each one to remove any contaminating substances and Spongiosa from the area of the Cavitas medullaris. For this work, the mill is fitted with a new disc (Orthodontros, milling head of plastic-diamond with 3-layer trianonlining, GD-No. 6830).

The bone samples are then mechanically ground into a fine meal with a vibration mill and sterile agate mortars in order to obtain the largest possible surface for subsequent lysis and extraction steps. The bone meal is then placed in a container with a screw-on top (Falcon BlueMax 2070) and mixed. From the sample size described, about 6–8 g of bone meal can be obtained.

The mix-and-clean method (MCM) of DNA isolation

1. Bone meal (0.2 g) is mixed with 500 μl of prewarmed (60°C) Mix I buffer [8.0% sucrose, 5.0% Triton

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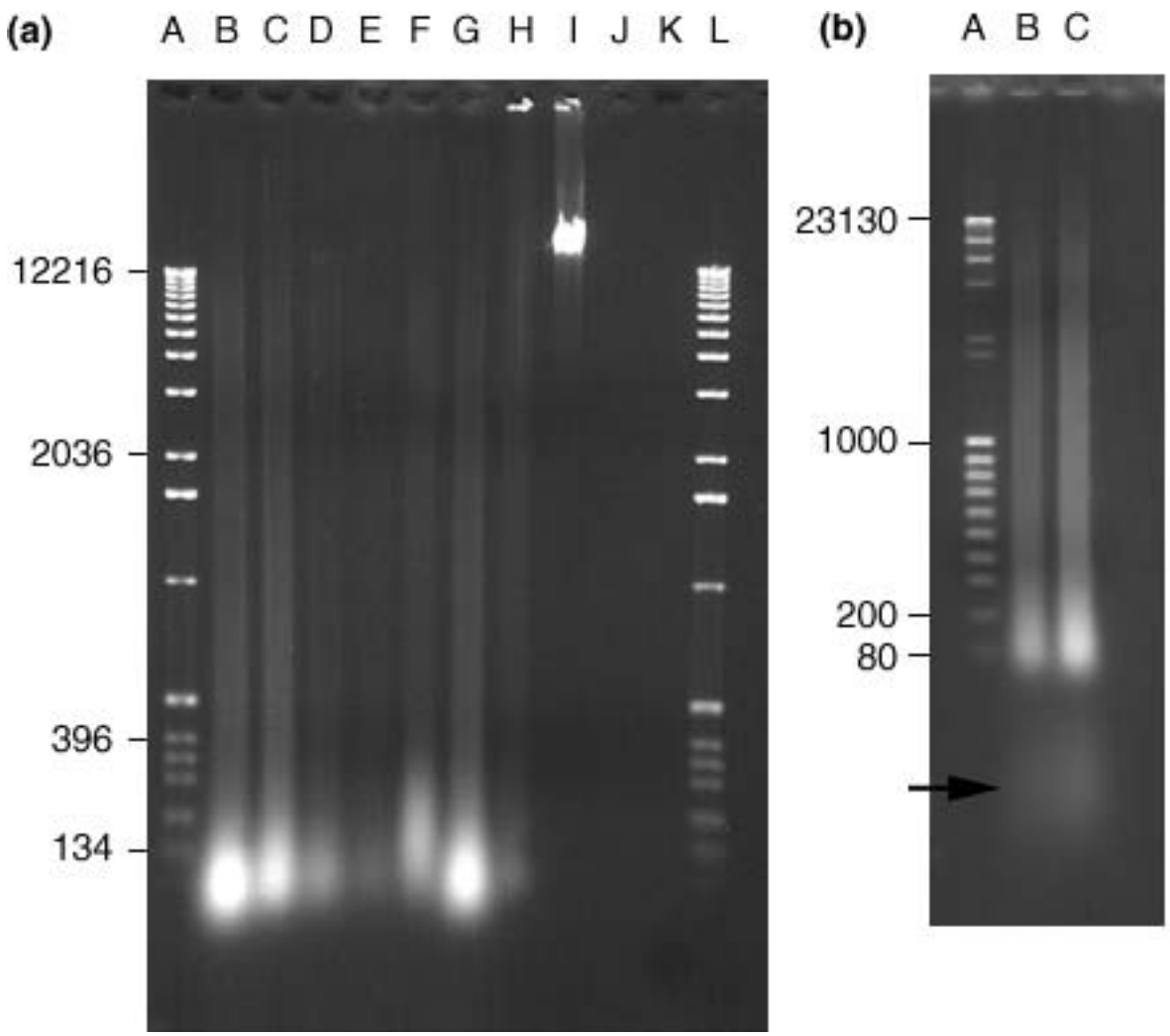

FIGURE 1. (a) Gel resolution of several DNA samples prepared from various bones with the mix-and-clean method (MCM). Lanes A and L, standard DNA markers 1 kb ladder (Boehringer Mannheim); lanes B–H, isolated material after a complete MCM procedure on ancient human bone samples including the removal of all humine acids; lane I, MCM-isolated DNA from a contemporary pig bone, showing that the method does not degrade the DNA; lane J, negative control, using fossilized bones (*Mammuthus primigenius*) with an estimated age of 35,000 years from which it was not possible to isolate DNA (racemization value: D/L-Leu > D/L-Ala < D/L-Asp > 0.08; Ref. 7), showing that there has been no contamination of the sample with foreign DNA during preparation; lane K, another negative check in which the full MCM preparation was carried out without a bone meal sample; no DNA was isolated. Each lane used 1 μl of the 15 μl DNA sample. All gels were stained with ethidium bromide. Sizes are given in basepairs (bp). (b) Lane A, a combined size-marker from 23,130 bp (λ*Hin*dIII) down to 80 bp 100 bp ladder (MBI/Fermentas); lanes B and C, MCM isolation up to step 7 of the recipe, using the same ancient bone samples from which the DNA is shown in lanes F and G in (a); the presence of contaminating humine acids is shown by the prominent green fluorescent strip (thick arrow) beneath the band smear of DNA.

X-100, 10 mM EDTA, 5 mM Tris-HCl (pH 8.0), 5 mM ammonium acetate, 2 mM magnesium acetate, 100 μl Sephadex G-50] in a reaction tube (Eppendorf) and vortexed vigorously for 1 min.

2. One vol. phenol is added to the suspension. To ensure efficient mixing of all components, the reaction vessel is placed on a shaker, model GS (Bachofer) at a setting of 220 rev/min for 5 h at a temperature of no more than 45°C.
3. Phase separation is carried out by centrifugation at 14,000 rev/min for 5 min in an eppifuge.
4. The aqueous layer is transferred to a new 1.5 ml vessel. After adding 1 vol. chloroform (approximately 500–600 μl), the mixture is vortexed for 2 min and then centrifuged once more as described in step 3.
5. The supernatant is placed into a new tube, mixed with 0.7 vol. propan-2-ol and 20 μg glycogen and carefully vortexed.

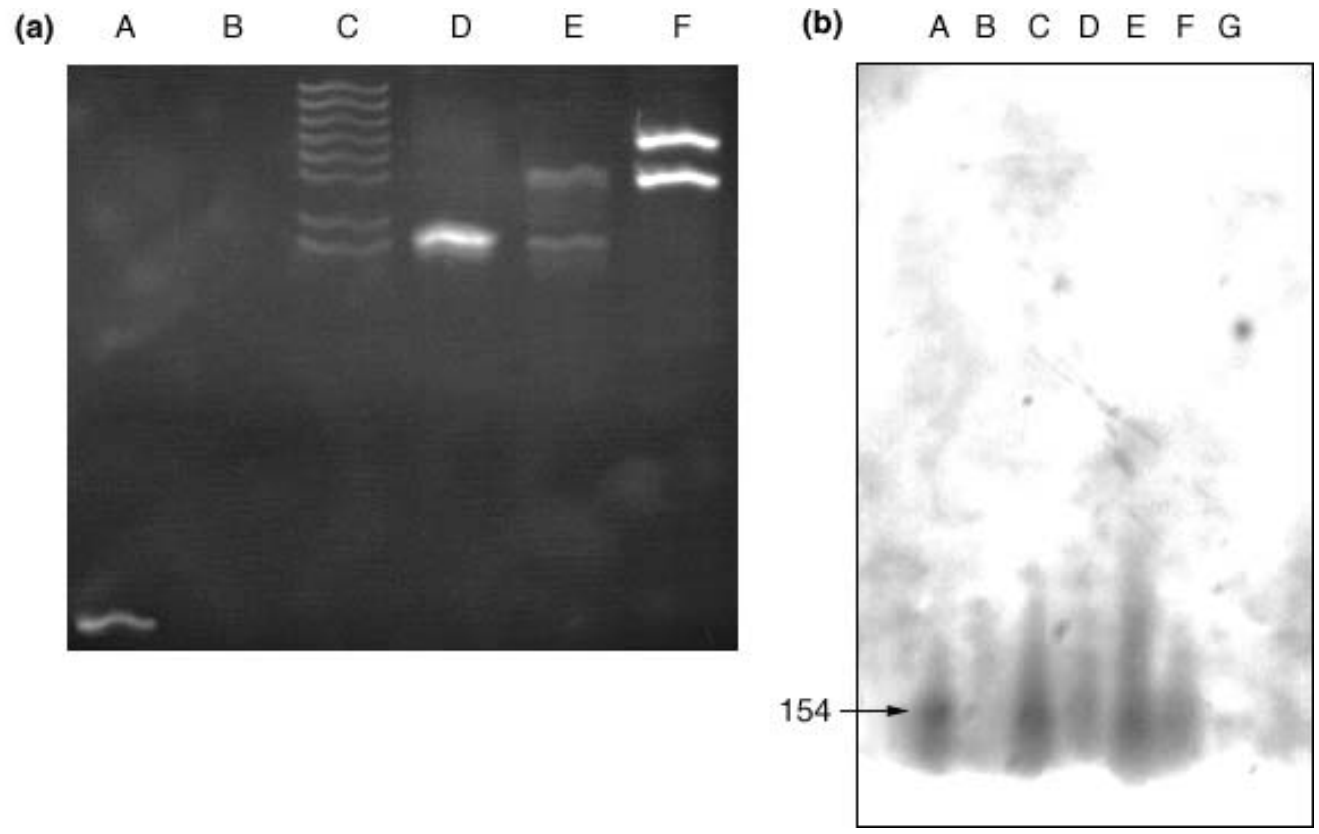

FIGURE 2. (a) Contamination tests to check DNAauthenticity. Separation ofPCR-products on a 9% polyacrylamide gel. Lane A, the PCR product of DNAfrom Fig. 1, lane E, generated by mt-specific primers 5984s and 60260px; lane B, same PCR conditions, but using the control *Mammuthus* extract (see Fig. 1, lane J), proving that no contamination with recent or ancient DNA occurred; lane C, CD4-allelic DNA standard marker (Serac); lanes D–F, STR-specific amplifications of the polymorphic CD4-locus Testkit zur DNA Typisierung (Serac) of isolated DNAs from Fig. 1a, lanes B, C, and F, using the same PCR mix and PCR program as used with mt-specific primers and performed according to the manual guidelines (Serac). (b) Southern blot hybridization of MCM-prepared DNA (lanes A–F) and degraded *Escherichiacoli* DNA (lane G). The 1% agarose gel was blotted onto positively charged nylon membranes (Genescreen) and the filter was subsequently hybridized with radioactively marked sonicated human placental DNA (Sigma). The figure serves as proof that the isolated DNA is of human origin.

6. Precipitation of the DNA is carried out at −20°C for 30 min.
7. The DNA is pelleted by centrifugation at 12,500 g at 4°C for 15 min.
8. The DNA pellet is air-dried and subsequently resuspended in 200 μl Mix II buffer (8% sucrose, 0.1% Triton X-100, 5 mM EDTA, 1.2 M NaCl).
9. In order to remove any humine acids, sugars and collagen structures efficiently, steps 5–8 are repeated once or twice, depending on the origin of the sample, but no more glycogen is added.
10. After these cleaning steps, the DNA pellet is washed for 15 min in 70% ethanol, vortexed briefly and centrifuged once more at 12,500 g at room temperature for 10 min.
11. The pellet is dried slowly at room temperature and then the DNA is dissolved in 10–15 μl TE (pH 7.2–8.0) or stored at −20°C for further processing.

The yield of re-isolated nucleic acids, which are now completely freed of humine acids (Fig. 1a), is up to 400 ng DNA per 0.1 g of bone meal. We have already used MCM-isolated prehistoric human DNA (1,300–1,500 years old) for the subcloning of fragments into plasmid vectors, and PCR reactions using STR-kit systems and contamination-sensitive Taq polymerases (Fig. 2a). Restriction digests and radioactive labelling of ancient probes for hybridization reactions with Southern and northern blots have also been performed successfully.

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Products Used

DNAaway: DNAaway from Molecular Bio-Products
1kb ladder: 1kb ladder from Life Technologies (Gibco BRL)
1 kb ladder: 1 kb ladder from Boehringer Mannheim
100 bp ladder: 100 bp ladder from Promega Corporation
nylon membrane: nylon membrane from Amersham Pharmacia Biotech
human placental DNA: human placental DNA from Sigma